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THE MOLECULAR ORGANIZATION OF THE ACTIVE CENTER OF MICROSOMAL CYTOCHROME P-450

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M. R. Borukayeva

Translation of: "O Molekulyarnoy Organizatsii Aktivnogo Tsentra Mikrosomal'nogo Tsitokhroma R-450",
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16. Abstract

The isocyanide spin label (ISL) induces differential optical spectra in microsomes which are characteristic for isocyanide derivatives of cytochrome P-450. This, in addition to the quantitative correlation between the content of P-450 heme groups of cytochrome P-450 in the microsomes and the bound ISL, and the close correlation between the intensity of induced ISL spectral changes of cytochrome P-450 and the degree of inhibition of its hydroxylating activity indicate binding of ISL to the heme group of P-450. The form of the ESR spectra of ISL indicate that the heme of cytochrome P-450, to which the label was bound, all located quite far apart from one another and do not form dimeric structures. In intact microsomes ISL are not replaced by cyanide ions, while the spectra of the label are not broadened by wetter soluble paramagnetic ions. Agents which convert cytochrome P-450 into the P-420 form (P-Chloromercuribenzoate, urea, deoxycholate) sharply increased the molecular mobility of ISL but have no influence on the degree of its aqueous phase. On the basis of the obtained result, one can conclude that the active center of cytochrome P-450 is a single heme group surrounded by nonpolar amino acid residues whose spatial arrangement relative to heme changes in a certain fashion during cytochrome interaction with substrates and under conditions of conversion of P-450 to P-420.

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THE MOLECULAR ORGANIZATION OF THE ACTIVE CENTER OF MICROSOMAL CYTOCHROME P-450

L. M. Raykhman, B. Annayev, V. S. Belova and M. R. Borukayeva[1]

Cytochrome P-450 is one of the components of a system of free (i.e., not /399* bound with phosphorylation) oxidation in the membranes of the endoplasmatic reticulum (microsomes), of the mitochondria of the adrenals and of certain bacteria [1]. Accomplishing the function of terminal oxidase, cytochrome P-450 directly participates in enzymatic hydroxylation, activating the molecular oxygen in such a fashion that it becomes possible to include it in various non-polar compounds of both endogenic and exogenic origin (steroid hormones, pharmaceutical substances, etc.) [1, 2].

The characteristic property of cytochrome P-450, which it must have by virtue of its name, is the arrangement of its Sorier bands of its complexes with CO in a longer wave region (450 nm) than in the case of the carbonyl derivatives of the majority of the other hemoproteins [3]. Cytochrome P-450 is relatively unstable and under the effect of various agents converts into another form — cytochrome P-420 with an "ordinary" Sorier band at 420 nm [4].

An interesting property of cytochrome P-450 is also the fact that many non-polar compounds which vary in their chemical nature are capable of bonding with it and cause qualitatively identical changes in its optical properties in the region of the Sorier band of the heme group, increasing optical density at 385-395 nm and decreasing optical density at 430 nm (the so-called first type of spectral changes) [5]. At the same time, the hydroxy- /400 lating substrates, causing similar changes, are linked in all probability not directly to the iron of the heme group but rather to a certain hydrophobic region, inasmuch as the formation of an enzyme-substrate complex in the microsomal hydroxylating system, as was shown in our preceding study, was primarily

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due to forces of hydrophobic interaction [6]. Certain nitrogen containing ligands cause spectral changes of a reverse character — they increase absorption at 420-430 nm and decrease optical density at 390 nm, which is caused by their formation of complexes of the hemochromogen type with the heme group of cytochrome [5]. However, in addition to such "hemochromogenic" changes, these substrates also cause spectral changes of the first type, which result in a stronger expression of the hemochromogenic spectra [7].

The reasons for qualitatively identical changes in the optical properties of cytochrome P-450 during its interaction with non-polar compounds having varying chemical natures, as well as the mechanism for conversion of cytochrome P-450 into cytochrome P-420 are not adequately clear. One of the possible explanations consists in the fact that these phenomena are linked with conformational transistions of cytochrome P-450, as the result of which there is a change in the mutual arrangement of the heme group and the adjacent regions of the protein part of the cytochrome.

A method of spin labels [8] was employed in this study for detecting possible molecular restructurings near the heme group of cytochrome P-450 during its interaction with substrates and under conditions of conversion of the P-450 form into the P-420 form. With this goal, an isocyanide spin label (ISL) [9] was synthesized; thanks to the presence in it of -N = C-groups, it is capable of specifically interacting with heme of cytochrome P-450 (in microsomes, besides P-450, there is also cytochrome B_5, but it does not interact with isocyanides [10]).

According to the ESR spectra of the iminoxyl part of the label, one can determine the frequency of its molecular conversion [8, 11]. This frequency depends, in a number of other factors, on the steric obstacles with respect to surrounding molecules, which makes it possible, with respect to a change in the frequency of conversion linked to heme of ISL, to make a judgement concerning change in spatial arrangement of heme and neighboring protein groups.

Method

The work was carried out on microsomes obtained from the liver of the rat after the Omura and Sato [3] method. For increasing the liver content of

cytochrome P-450 and for increasing the activity of the microsomal hydroxylating system, the animal was preliminarily administered phenobarbital in the amount of 10 mg of preparation per 100 g of live weight, daily, for a period of 5 days. The concentration of microsomal protein was measured by the biuretic method [12].

The content of cytochrome P-450 in the microsomes was determined in the following manner. Both cuvettes of the DSF-1M differential spectrophotometer were filled with the microsomal suspension containing 2 mg protein per 1 ml, and following establishment of a zero line, one of the cuvettes was saturated with carbon monoxide. Then, dithionite was added to both cuvettes and the differential spectrum was recorded. The concentration of cytochrome P-450 was calculated, using the value of the difference of the coefficients of extinction at wavelengths of 450 and 490 nm, and was equal to 91 $mM^{-1} \cdot cm^{-1}$ [3].

Hydroxylating activity of the microsome was determined according to the oxidation rate of 1-^{14}C-octadecane, as was described in the preceding study [6].

ISL was synthesized according to the earlier described method [9]. Precipitation of the label on the microsomes was carried out in the following manner. 1 mM ISL was added to the suspension of microsomes (concentration of microsomal protein 5 mg/ml) in a mixture of tris-KCl 50 mM KCl and 50 mM tris--buffer, pH 7.5) and was incubated 30 minutes at 25°. In the experiments, for processing the microsomes with P-chlormercuribenzoate (PCMB) (2 mM), urea (8 M) and deoxycholate (4%), the microsome suspension was additionally incubated with these reagents, in the indicated concentrations, for a period of 10 minutes at 25°. For removing the unbound label, the suspension of microsomes was centrifuged at 1 to 2° for a period of 2 hours at 80,000 g. The supernatant, containing the unbound label, was removed, while the precipitate was resuspended in a cold mixture of tris-KCl (which also contained PCMB, urea, or deoxycholate in the tests which employed these reagents) and were once again centrifuged for two hours at 80,000 g. This procedure was repeated twice.

The precipitate of microsomes obtained following the second centrifuging /401 was resuspended in a certain quantity of medium having the same composition as that in which the incubation was conducted, but without ISL, so that the

concentration of microsomal protein comprised 40 to 50 mg/ml. The obtained suspension was placed in fine, glass pipettes if the measurements of ESR spectra were conducted at room temperature, or in polyethylene pipettes in case the measurements were conducted at the temperature of liquid nitrogen. The ESR spectra were recorded in the EPR-2 radiospectrometer. The concentration of label in the microsomes was determined by comparing the integral intensity of the ESR signal of the label samples at the temperature of liquid nitrogen with the intensity of the signal (at the same temperature) of a standard solution having a known concentration of the iminoxyl radical.

For determining the content of unbound label, part of the suspension prepared for ESR measurements, after being stored for a certain period of time at room temperature or in an ice bath, was centrifuged at a temperature of 1 to 2° for a period of 2 hours at 80,000 g and then the ESR spectra of the supernatant and precipitate were recorded, preliminarily resuspending them so that the concentration of protein in the suspension was 40-50 mg/ml. In this case, even in the event of storing the samples up to 1 hour at room temperature (a more prolonged incubation at room temperature was inexpedient in view of the possible destruction of cytochrome P-450), or up to 8 hours at a temperature of 0-2° in the supernatant, an extremely weak ESR signal was detected which corresponded to the concentration of label of no more than 10^{-6} M, while in the resuspended precipitate one observed a quite intensive signal which corresponded to a label concentration of $5 \cdot 10^{-5} - 10^{4}$ M. Hence, the free label whose contents comprised only a slight fraction of the content of a bound ISL could not make a significant contribution to the ESR spectrum of the spin label microsomes.

The frequency of conversion of the label was calculated in accordance with the study [11] according to the formulae

$$v_{+1} = \frac{2 \cdot 10^8}{\left(\sqrt{\frac{I_0}{I_{+1}}} - 1\right)\Delta H_0} \text{ sec}^{-1}, \quad v_{-1} = \frac{3{,}25 \cdot 10^8}{\left(\sqrt{\frac{I_0}{I_{-1}}} - 1\right)\Delta H_0} \text{ sec}^{-1}$$

where v_{+1} and v_{-1} are the frequencies of conversion of the label calculated in accordance with the low field and high field components of the ESR spectrum of

the label, I_{+1}, I_0 and I_{-1} are the amplitudes of low field, central, and high field components, ΔH_0 is the width of the central component. In addition to v, parameter ε was also determined:

$$\varepsilon = \left(\sqrt{\frac{I_0}{I_{+1}}} - 1\right) \Big/ \left(\sqrt{\frac{I_0}{I_{-1}}} - 1\right).$$

Magnitude ε, according to the study [13], reflects the degree of asymmetry of the molecular surroundings of the radical. It is necessary to bear in mind, however, that in complex biological systems conditions for variability of theories [11, 13] are not always strictly satisfied and v and ε should be viewed simply as certain effective parameters according to whose magnitude of change one can form a judgement concerning the degree of expression of conformational transitions.

Results of the Investigations

Figure 1 shows the differential spectra of microsomes processed with ISL. Adding ISL to the aerated microsomes increases the optical density at 435 nm and decreases it at 400 nm. In the case of microsomal preparations reduced by NADP·N or dithionite, ISL causes the appearance of 2 maxima at 435 and 455 nm with a corresponding height of peaks (from the zero line) at 455 nm and 435 nm of nearly 1.3. Such spectra are characteristic of isocyanide derivatives of cytochrome P-450 [9], and indicate that ISL is significantly bound to the heme group of this cytcochrome.

ISL is also capable of effectively inhibiting hydroxylation of substrates in microsomes indicates bonding of the label with the heme of cytochrome P-450, which is an active center of microsomal hyroxylation system; one also observes a good correlation between the degree of inhibition and the degree of expression of induced ISL of optical changes of cytochrome P-450 with various concentrations of the label (Figure 2). The curve of the dependency of the rate of hydroxylation of 1-^{14}C-octadecane on the concentration of ISL straightens well in the coordinates $\left\{[1]; \frac{1}{v_i}\right\}$ in accordance with the equation for nonconcurrent inhibition /402

$$\frac{1}{v_i} = \frac{1}{V_m}\left(1 + \frac{K_m}{[S]}\right)\frac{[I]}{K_i} + \frac{1}{V_m}\left(1 + \frac{K_m}{[S]}\right),$$

where [I] - concentration of inhibitor (ISL), [S] – concentration of substrate (octadecane) v_i – rate of reaction at a given concentration of inhibitor, V_m – maximal rate of reaction in the absence of inhibitor, C_m – Mikhayelis constant for the substrate, C_i – inhibition constant.

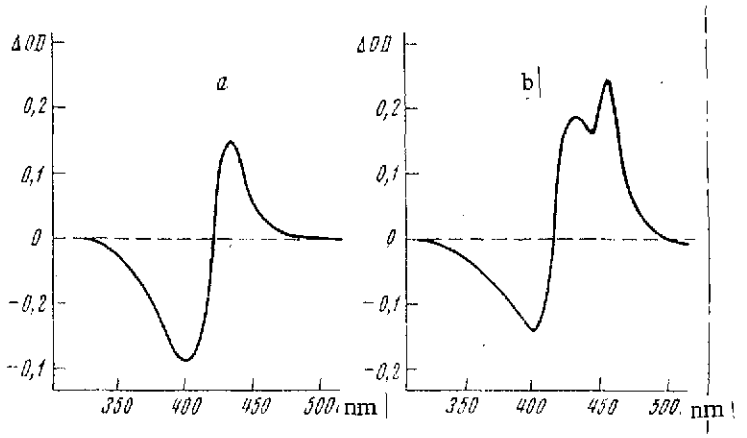

Figure 1. Differential Spectra of Microsomal Suspension When ISL is Added: a, ISL added to the microsomes preliminarily oxidized by 20 minute aeration; b, ISL added to microsomes reduced by 5 mM NADP•N. (The medium contained 50 mM tris-buffer (pH 7.5), 50 mM KCl and 5 mM $MgCl_2$. The concentration of microsomal protein 2 mg/ml; concentration of ISL 0.2 mM. The spectra were recorded at room temperature).

At the same time, the curve of dependence of the intensity of the differential spectra on the concentration of ISL straightens within the coordinates $\{[I]; [I]/OD\}$ in accordance with a modified Laynuivera-Burke equation:

$$\frac{[I]}{OD} = \frac{K_s}{OD_{max}} + \frac{[I]}{OD_{max}},$$

where the rates of the reactions are replaced by the intensities of spectral changes OD. C_s is the "optical" analog of C_m and has the meaning of the concentration of label at which the intensity of the spectral changes coincide to half the maximal. As one can see from Figure 3, the points of both curves from Figure 2 in the corresponding coordinates lie nearly along a single line, indicating the closeness of values C_i and C_s, comprising nearly 10^{-5} M.

Figure 4 shows the ESR spectrum of a frozen preparation of the spin labelled microfilms at the temperature of liquid nitrogen. The shape of the spectrum is very near in form to the ESR signal of the added solution of iminoxyl radical in the spirit at the same temperature. In accordance with the study [14], on the basis of such a shape of spectra one can conclude that iminoxyl radicals in the spin labelled microsomes are located apart from each other at a distance no less than 18-20 Å. The intensity of the ESR signal of the

spin labelled microsomes corresponds to the concentration of radicals in a sample $8.5 \cdot 10^{-5}$M or 1.75 nmol on 1 mg of microsomal protein. This magnitude, taking into account the accuracy of determining absolute concentrations by the ESR method (nearly 10%), adequately well coincides with the heme content of P-450 in microsomes determined according to the differential spectra of CO in the atmosphere, which, in our preparations, corresponded on the average to 1.65 nmol heme of cytochrome P-450 for 1 mg of microsome protein. /403

Linking ISL to the heme groups of cytochrome P-450 according to a stoichiometry of 1:1, in addition to the large distance between molecules of ISL linked with the heme groups, does not support the hypothesis [15, 16] concerning dimeric organization of the active center of cytochrome P-450 in the form of 2 closely arranged hemes. It is also necessary to give some attention to the typically hyperbolic shape of the "absorption" curve of cytochrome P-450 of ISL (Figure 2), without any signs whatsoever of S-shape. This also indicates the absence of a noticeable interaction between the heme groups in cytochrome P-450. Hence, from these data it follows that the active center of cytochrome P-450 is a single heme.

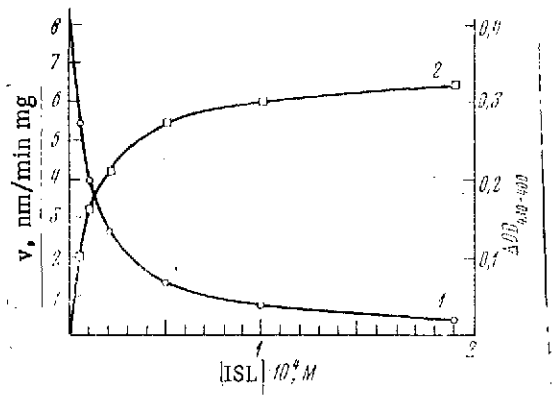

Figure 2. Dependence of Hydroxylating Activity of Microsomes (1) and Intensity of Induced ISL of Differential Spectra of Microsomes (2) on the Concentration of ISL: v, rate of hydroxylation of 1-^{14} C-octadecane (concentrations of octadecane 1 mM); $\Delta OD_{430-400}$, difference in optical densities at 430 and 400 nm in differential spectra of aerated microsomes.

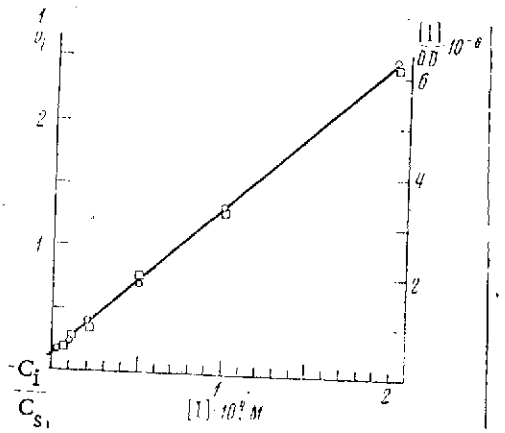

Figure 3. Dependence of Hydroxylating Activity of Microsomes on Concentrations of ISL in the Coordinates $\{[I]; 1/v_i\}$ (○) and the Intensity of Differential Spectra of the Microsomes on the Concentration of ISL in the Coordinates $\{[I]; [I]/OD\}$ (□) (Explanations in text).

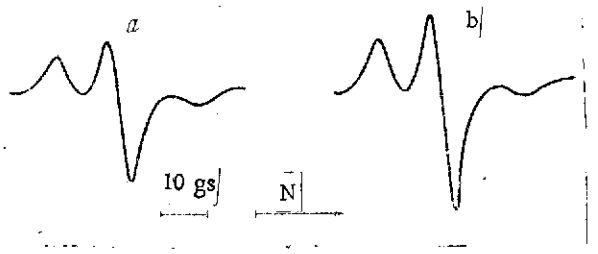

Figure 4. Spectra of ESR ISL in Microsomes (a) and in the Solution of Iminoxyl Radical in Spirit (b) At the Temperature of Liquid Nitrogen. The concentration of microsomal protein in the sample is 50 mg/ml, the concentration of spirit solution of iminoxyl radical is $1.25 \cdot 10^{-4}$ M.

We do not exclude the possibility, however, that a molecule of cytochrome P-450 consists of several subunits, each of which contains heme, although the s-shape is absent and does not indicate such an hypothesis.

Figure 5 shows spectra of ESR ISL in various microsomal preparations taken at room temperature. The spectrum of ISL in intact microsomes is characterized by a significant inhibition, however the frequency of conversion of ISL bound to cytochrome P-450 noticeably increases upon adding hexabarbital or aminopyrine — substrates which bond to cytochrome P-450 and which cause primary spectral changes. The same effect is observed /404 when processing the microsomes with thiol reagent, PCMB, which inhibits hydroxylating activity [17] and converts cytochrome P-450 into cytochrome P-420 [18]. There is a particularly strong increase in the mobility of ISL (more than threefold if one calculates according to v_{-1} and more than fifteenfold if one calculates according to v_{+1}) which is caused by deoxycholate and urea, which, in a manner similar to that of PCMB, induced the conversion of P-450 into P-420. This effect cannot be explained merely as the result of deception of the bound label under the influence of urea and deoxycholate. Experiments for determining the content of free ISL in such preparations (i.e., ISL detected in the supernatant following centrifuging of the spin-labelled microsomal suspension for a period of 2 hours at 80,000 g and at a temperature of 1-2°) showed that although the content of free ISL during incubation with urea and deoxycholate increases in comparison with the intact preparations, but nevertheless does not exceed 15-20% of the content of bound label. Suspending the microsomes in the medium with a high ion strength noticeably decreases the mobility of the label. The changes in the spectra of the ESR of microsomes shown in Figure 5, with various agents, cannot be caused by the interaction effect between the unpaired electrons

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of the label and the heme iron. Such spin-labelled interaction can have as its result only a uniform expansion of all spectral lines of the ESR label. In the ESR spectra of various preparations of spin-labelled microsomes shown in Figure 5, one observes a change in the relationships of width (and, correspondingly, in amplitude) of various spectral components, which doubtlessly indicates a change, in fact, in the frequency of molecular conversion of the label [10].

The numerical values of the parameters of conversion v_{+1} and v_{-1} are shown in the table. The fact attracts attention that although the tendency to change of v_{+1} and v_{-1} for various microsomal preparations is the same, their magnitudes coincide (within the limits of measurement error) only for microsomes processed with urea and deoxycholate. In other cases, in which the label was more inhibited, v_{+1} and v_{-1} strongly differed from each other, which was probably caused by the fact that there was a quite strong anisotrophy of the molecular surroundings of the label (v_{+1} and v_{-1} coincide only in an isotrophic medium), which does not permit its conversion in all directions in an identically free fashion. /405

The anisotrophy of the molecular surroundings of the label is more directly characterized by parameter ε. From the table one can see that the higher the frequency of conversion of the label, the lower the value of ε. It is minimal for microsomes processed by deoxycholate and urea, where values v_{+1} and v_{-1} are maximal and coincide. Apparently, the increase in mobility of ISL, bound with the heme of cytochrome P-450 is achieved as the result of a certain separation from the heme of the neighboring protein groups, which makes the molecular surroundings of the label more symmetrical (decreases parameter ε).

Figure 6 shows the effect of potassium cyanide on ESR spectra of ISL in microsomes. One would expect that cyanide, having a high affinity for iron porphyrins, would displace the ISL from the heme group into the surrounding medium. This should lead to a sharp increase in the mobility of the ISL to values characteristic for aqueous solutions of the label. As one can see from Figure 6, even relatively high values of concentration of cyanide (up to 10 mM) have no effect on the ESR spectra of ISL in intact microsomes and do not decrease the degree of its bonding with the heme of cytochrome P-450. The influence of cyanide is also not manifested in the presence of hexabarbital

and during suspension of the microsomes in a medium with high ion strength. However, during incubation with cyanide of microsomal preparations processed with urea or deoxycholate, the mobility of the label sharply increases to values characteristic for aqueous solutions. This indicates desorption of the label from the heme group, which is also supported by its complete removal during subsequent washing of the preparations. One can conclude from these data that in intact microsomes both without the hydroxylating substrate and in its presence, ISL together with the heme group to which it is bonded are in the hydrophobic surroundings and are not accessible to the cyanide dissolved in the water. However, processing with urea and deoxycholate leads to such changes in the structure of the cytochrome that as a result of which the heme group begins to come into contact with the aqueous medium in which the cyanide ions are dissolved, and the latter obtain, hence, the possibility of dislodging the ISL from the heme group of cytochrome P-450.

NUMERICAL VALUES OF PARAMETERS v_{+1} v_{-1} AND ε IN VARIOUS MICROSOMAL PREPARATIONS

Preparation	$v_{+1} \cdot 10^8$, sec^{-1}	$v_{-1} \cdot 10^8$, sec^{-1}	ε
Intact microsomal preparations	1,8±0,1	9,9±0,2	0,345±0,010
Aminopyrine (2 mM)	5,7±0,4	17,3±0,6	0,195±0,015
Hexabarbital (2 mM)	6,4±0,5	19,3±1,1	0,200±0,030
PCMB (2 mM)	8,7±0,4	21,8±1,4	0,165±0,015
Urea (8)	30,3±3,2	33,4±3,5	0,060±0,010
Deoxycholate (4%)	32,5±3,1	30,5±3,2	0,055±0,010
Microsomes in 0.5 M phosphate buffer	1,2±0,1	6,9±0,2	0,345±0,010

Commas indicate decimal points.

The influence of water soluble paramagnetic ions on ESR spectra of ISL in microsomes was also investigated. The paramagnetic anion of ferricyanide broadens the spectral lines of ESR above the iminoxyl radical without a change in its integral intensity, while the magnitude of broadening of δ is directly proportional to the concentration of the paramagnetic [19]. One can write the following empirical equation

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$$\delta = [\Delta H_{+1} - (\Delta H_{+1})_0]/(N_{+1})_0 = cC,$$

where ΔN_{+1} and $(\Delta N_{+1})_0$ are the width of the low field component of the spectrum, respectively, in the presence of ferricyanide and without it; C — concentration of the paramagnetic mol/l; c — empirical constant of broadening in the measurement l/mol. Under otherwise equal conditions, c is maximal in the aqueous solution of spin label and decreases if the label becomes poorly accessible for the paramagnetic dissolved in the water.

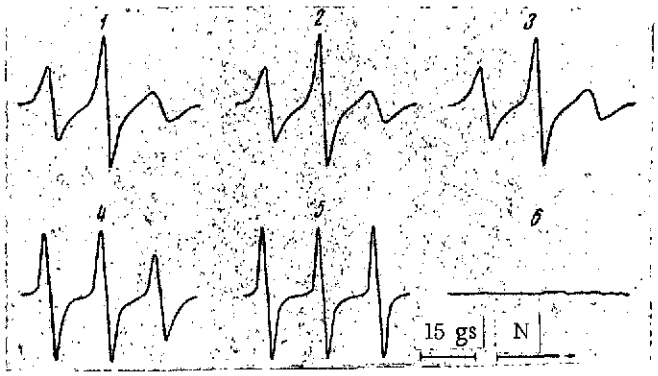

Figure 5. Spectra of ESR ISL in Various Microsomal Preparations at Room Temperature: 1, intact microsomes; 2, microsomes in the presence of 2 mM hexabarbital; 3, microsomes processed with 2 mM PCMB; 4, microsomes processed with 8 M urea; 5, microsomes processed with sodium deoxycholate (4%); 6, microsomes suspended in medium with a high ion strength (0.5 M phosphate buffer pH 7.5).

As one can see from Figure 7, the ESR spectrum of the label bound to cytochrome P-450 does not undergo broadening because of the ferricyanide. This means that ISL bonded to the heme group of cytochrome P-450 is apparently located in the hydrophobic surroundings and is deprived of the possibility of the coming into contact with the aqueous medium where the ferricyanide ions are located. Hence, these data are in agreement with the results of experiments cited above pertinent to the effect of cyanide on the degree of bonding of ISL with the heme group of cytochrome P-450. Hexabarbital, noticeably increasing the mobility of ISL, has almost no influence on the degree of its contact with the aqueous phase. PCMB, increasing the frequency of molecular conversion of ISL to approximately the same degree as hexabarbital, in differentiation from the latter, significantly increases the accessible of ISL for ions of ferricyanide. The strongest magnitude of c increases with processing of the microsomes by urea and deoxycholate, which also increase the mobility of ISL to the greatest degree.

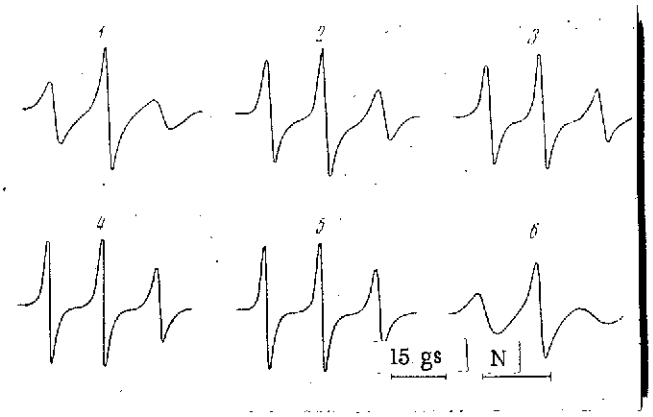

Figure 6. The Influence of Cyanide on the ESR Spectra of ISL in Intact Microsomes and in Microsomes Processed by Deoxycholate: 1, intact microsomes without cyanide; 2, the same microsomes following incubation with a 10 mM solution of potassium cyanide (20 minutes, 30°); 3, microsomes incubated with cyanide following washing; 4, processed by 4% solution deoxycholate in the absence of cyanide; 5, processed by 4% solution deoxycholate following incubation with 10 mM cyanide (20 minutes, 30°); 6, microsomes processed by deoxycholate and incubated with cyanide following washing.

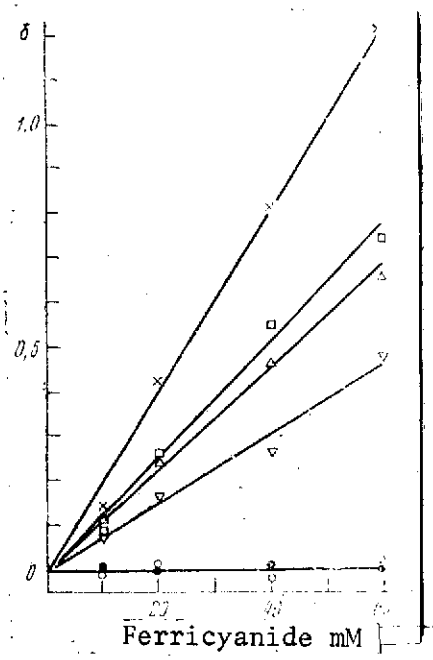

Figure 7. The Effect of Ferricyanide on Broadening of ESR Spectra of ISL in Various Microsomal Preparations: intact microsomes (○); microsomes in the presence of 2 mM hexabarbital (●); microsomes processed by 2 mM PCMB (▽); microsomes processed by 8 M urea (△); microsomes processed by 4% deoxycholate (□); aqueous solution of ISL (x).

A Discussion of the Results

In this study we have established the following: 1) the capacity of ISL to induce differential optical spectra in microsomes characteristic for isocyanide derivatives of P-450; 2) a close correlation between the intensity of induced ISL spectral changes and the degree of inhibition of ISL hydroxylating activity of the microsome with various concentrations of label; 3) a quantitative agreement between the content of heme groups of cytochrome P-450 in microsomes and the bound ISL, which indicates the bonding of ISL with the heme group of cytochrome P-450.

From the shape of the ESR spectra of ISL in microsomes frozen in the preparations one can conclude that the heme of cytochrome P-450 are located

quite far apart from each other. At the same time, such facts as the incapacity of water soluble cyanide to displace ISL from the heme groups of cytochrome P-450 in intact microsomes, and the absence of a broadening of ESR lines of ISL dissolved in water by ions of ferricyanide indicate that the heme group of /407
cytochrome P-450 with the ISL bonded to it is not in contact with the aqueous phase. This apparently means that the heme group of cytochrome P-450 is in a close hydrophobic "band", formed by the nonpolar amino acid residues which wash against the heme, and, possibly, by some other nonpolar groups (for example, lipids which form with a molecule of cytochrome, a lipoprotein complex). The fact that the band is quite "close" is indicated by the relatively low frequency of molecular conversion of the ISL located in it.

The arrangement of the heme group in the close hydrophobic band is, apparently, one of the reasons for the significant stability of the complex of cytochrome P-450 with ISL, which, as experiments with washing the spin-labelled microsomes show, dissociates to an extremely small degree even over the course of an hour at room temperature or over a period of 8 hours at the temperature 0-2°. To confirm the great role which can be played in the stable complex of P-450 with ISL by the hydrophobic interaction of the tetramethylpiteridine fraction of the label with nonpolar amino acid residues which surround the heme, one can refer to the data of a study [20], where it was shown that the constant of dissociation of the complex of oxidized P-450 with isocyanides decreases by two to three orders during the transition from methyl- to tri-butyl- and phenyl isocyanides. A comparison of cyanide complexes of cytochrome C and hemoglobin can also serve as a visible illustration of the value of protein groups for stability of the complex of heme with ligands. In the first instance, the time of semidissociation of the complex at room temperature is several hours [21, 22], while the cyanide complexes of hemoglobin dissociate in fractions of a second [23].

An increase in the molecular mobility of ISL in the spin-labelled microsomes with a simultaneous decrease in the asymmetry of its molecular surroundings under the influence of the hydroxylating substrate is linked apparently with the fact that the latter, as it were, somewhat "expands" the hydrophobic band around the heme, separating neighboring groups from it, but during this process

the band remains "hermetic", i.e., inaccessible for the aqueous phase. This leads one to the idea that nonpolar substrates, being bound with cytochrome P-450, introduced between the hydrophobic groups' protein which forms the walls of the hydrophobic band, in this manner somewhat expanding them and increasing the "internal volume" of the band, at the same time preserving its "hermetic seal."

PCMB, and especially urea and deoxycholate also increase the mobility of ISL and additionally, "unseal" the band, making the heme group with the bonded ISL accessible for water soluble ions. Data obtained in recent years indicate the possibility of a thiol group located near the heme [24]. In the case of arrangement of this group within the hydrophobic band, interactions of PCMB with it, it would seem, must be made more difficult, if one takes into account the fact, for example, that the heme located within the band is poorly accessible for ions of cyanide and ferricyanide. The effect of PCMB must be either related to the fact that the "walls" of the hydrophobic band surrounding the heme are more permeable for PCMB than for ions of ferricyanide (it is well known, for example, that phospholipid membranes are a great deal more permeable for organic cations and anions than even for small inorganic ions [25]), or to the fact that PCMB links not with the thiol group which is located within the hydrophobic band, but with some other (one or more) thiol groups located outside the hydrophobic band but near the amino acid residues which form its walls. Introducing the molecule of PCMB could disrupt the spatial arrangement of the latter and in this way lead to a partial dehermetization of the band.

The effective deoxycholate is apparently linked to the fact that it, being /408
a detergent, disrupts the hydrophobic bonds between the nonpolar groups which form the walls of the hydrophobic band. Urea can lead to similar consequences due to the destruction of the protein molecule, which it causes. An increase in the frequency of conversion of ISL with an increased ion strength of the medium in which the microsomes are suspended can be related to the fact that high ion strength stabilizes the hydrophobic interactions [26]. As the result of this the nonpolar groups forming the walls of the hydrophobic band press more closely against each other, in this way increasing the structural obstacles for the ISL located within the band.

The fact attracts attention that a significant increase in the molecular mobility of ISL bound to cytochrome P-450 with simultaneous dehermetization of the hydrophobic band around the heme occurs under those conditions (processed by PCMB, urea, deoxycholate), under which cytochrome P-450 changes into the P-420 form [3, 4]. The presence of a maximum at 450 nm in the carboxyl derivative of P-450 is an unusual property of this cytochrome. As modeling experiments show, such a long wave change can be caused by the formation of a molecular complex stabilized by hydrophobic bonds between the porphyrin ring of heme and compounds with delocalized p-electrons [16]. These can be, for example, aromatic amino acid residues of the protein fragment of cytochrome or certain other unidentified compounds with conjugate bonds.

It is natural to expect that agents which cause separation of the "aromatic" region from the heme (i.e., containing aromatic amino acids or other compounds which cause a long wave change in the Sorier band) and which thus convert P-450 into P-420 should decrease, as well, the structural obstacles for molecular conversion of ISL bound to heme, and thus increase its mobility in addition to "dehermetizing" the hydrophobic band around the heme.

For understanding the mechanism of the functioning of cytochrome P-450, the capacity of hydroxylating substrates (hexabarbital, amino pyrine) to increase the mobility of ISL during bonding with cytochrome P-450 is of great interest; this indicates conformation changes induced by the substrate consisting in separating certain protein ligands from the heme. This is probably also caused by the transition of cytochrome P-450 from the low and high spin state with complex formation with the substrate, which was found in a study [24].

The separation of certain ligands from an atom of iron can also lead to a decrease in the electron density in the latter, and, consequently, increase the oxidizing reducing potential of cytochrome, in this manner activating its reduction by preceding electron carriers. Actually, as has been shown in a study [27], hexabarbital and other substrates which cause spectral changes of the first type hasten reduction of the cytochrome P-450.

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